Mitochondrial dysfunction and Purkinje cell loss in autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS)

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Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is a childhood-onset neurological disease resulting from mutations in the *SACS* gene encoding sacsin, a 4,579-aa protein of unknown function. Originally identified as a founder disease in Québec, ARSACS is now recognized worldwide. Prominent features include pyramidal spasticity and cerebellar ataxia, but the underlying pathology and pathophysiological mechanisms are unknown. We have generated an animal model for ARSACS, sacsin knockout mice, that display age-dependent neurodegeneration of cerebellar Purkinje cells. To explore the pathophysiological basis for this observation, we examined the cell biological properties of sacsin. We show that sacsin localizes to mitochondria in non-neuronal cells and primary neurons and that it interacts with dynamin-related protein 1, which participates in mitochondrial fission. Fibroblasts from ARSACS patients show a hyperfused mitochondrial network, consistent with defects in mitochondrial fission. Sacsin knockdown leads to an overly interconnected and functionally impaired mitochondrial network, and mitochondria accumulate in the soma and proximal dendrites of sacsin knockdown neurons. Disruption of mitochondrial transport into dendrites has been shown to lead to abnormal dendritic morphology, and we observe striking alterations in the organization of dendritic fields in the cerebellum of knockout mice that precedes Purkinje cell death. Our data identifies mitochondrial dysfunction/mislocalization as the likely cellular basis for ARSACS and indicates a role for sacsin in regulation of mitochondrial dynamics.

Drp1 | neurodegenerative disease | neuromuscular disease | Parkinson's disease

ARSACS (Online Mendelian Inheritance in Man: 270550) is a childhood-onset, autosomal recessive spastic ataxia originally identified in the Charlevoix and Saguenay regions of Québec. First described in 1978 by Bouchard et al. (1), ARSACS is clinically characterized by spasticity, ataxia, polyneuropathy, retinal changes and in some cases late cognitive decline. Patients demonstrate an unsteady gait and experience frequent falls as they learn to walk. Québec patients never walk properly, are wheelchair-bound on average by age 41, and their life expectancy is decreased to 51 years (2, 3). Progressive cerebellar atrophy is a prominent feature of ARSACS (4, 5), and in the two published autopsies, loss of cerebellar Purkinje cells was documented and found to be more severe in the older case (2, 6). These observations suggest that progressive death of Purkinje cells underlies the evolution of the ataxia although this hypothesis has not been tested experimentally. ARSACS is caused by mutations in the *SACS* gene, with the first two mutations identified in Québec patients in 2000 (7). Since then, >100 separate mutations have been identified from patients in 13 countries around the world, and interest in the disease continues to grow (5, 8–10). In fact, after Friedreich's ataxia, ARSACS may be the most common form of recessive ataxia (9).

The *SACS* gene encodes sacsin, a multimodular protein of 4,579 amino acids, one of the largest known proteins in the human genome. From N- to C-terminal, sacsin is composed of a ubiquitin-like domain that binds to the proteosome (11), three large sacsin repeat regions that may have an Hsp90-like chaperone function (12, 13), an XPCB domain that binds to the Ube3A ubiquitin protein ligase (14), a DnaJ domain that binds Hsc70 (11, 12), and a higher eukaryotes and prokaryotes nucleotide-binding domain that mediates sacsin dimerization (15). The nature of these modules suggests that sacsin may operate in protein quality control. However, the functional role of sacsin and the pathophysiological consequences of its dysfunction have not been examined.

In a previous study (11), we demonstrated that sacsin is predominantly cytoplasmic with a mitochondria component. Mitochondria are present in all eukaryotic cells (erythrocytes excluded) and have vital functions including biosynthesis of amino acids and steroids, calcium buffering, and notably, ATP production (16). Mitochondria are highly dynamic structures that constantly change their morphology through membrane fission and fusion. Mitofusins 1 and 2 and Opa1 are GTPases on the mitochondrial outer and inner membrane, respectively, and are responsible for fusion (17). Dynamin-related protein 1 (Drp1) is a large GTPase required for mitochondrial fission (18–21). These processes, collectively called mitochondrial dynamics, are important aspects of cellular quality control, and defects have detrimental consequences on cells including the supply of energy (22). Neurons are particularly sensitive to changes that disturb mitochondria, and determining how defects in mitochondrial function and dynamics lead to neuronal cell pathology is extensively studied in the field of neurodegenerative diseases (22–27).

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Here, we provide functional characterization of sacsin. Using cell lines, patient fibroblasts, primary neuron cultures, knockout (KO) mice, and organotypic brain slice cultures from the mice, we demonstrate that sacsin localizes to mitochondria and that targeted disruption of sacsin leads to alterations in mitochondrial morphology and function, impairs the organization of dendritic arbors, and causes neuronal cell death. These data suggest that ARSACS is caused by mitochondrial dysfunction and indicate that the disease shares some pathophysiological features with major neurodegenerative diseases with mitochondrial involvement including Alzheimer's, Parkinson's, and Huntington's diseases, thus ultimately aiding in the identification of therapeutic targets.

Results

Sacsin KO Mice Display Age-Dependant Loss of Purkinje Cells. Mutations in the *SACS* gene were determined to be causative of ARSACS in 2000 (7), yet in the intervening decade, there has been little progress in understanding the underlying pathology of the disease. For example, it is even unclear whether progressive neuronal death in the cerebellum is responsible for evolution of the ataxia, the major symptom, or whether the disease is predominantly developmental (28). To explore the neurodevelopmental and neurodegenerative aspects of ARSACS, a transgenic KO mouse model was generated in which most of the gigantic 12,794 bp exon 10 of *SACS* was replaced with an IRES-βGal cassette (Fig. S1*A*). Genomic PCR reveals the presence of the transgene in the offspring of the founders (Fig. S1*B*). Loss of sacsin message is confirmed by RT-PCR (Fig. S1*C*) and loss of protein is seen by Western blot (Fig. S1*D*) and immunofluorescence of brain regions (Fig. S1 *E*–*G*). Viable null animals are born with classical Mendelian inheritance. At the time of birth, there are no obvious phenotypic changes and gross brain morphology appears essentially normal. However, as the animals age, there is a notable loss of Purkinje cells in the cerebellum of the KO mice compared with wild-type littermates (Fig. 1). The loss of Purkinje cells is seen as early as 120 d (Fig. 1*A*), although it is not yet statistically significant (Fig. 1*B*), but becomes highly significant ($P < 0.001$) at 200 d (Fig. 1*C* and Fig. S1*H*). These data support the autopsy findings that Purkinje cell loss is an important aspect of the pathology and indicate that this process is primarily degenerative rather than developmental.

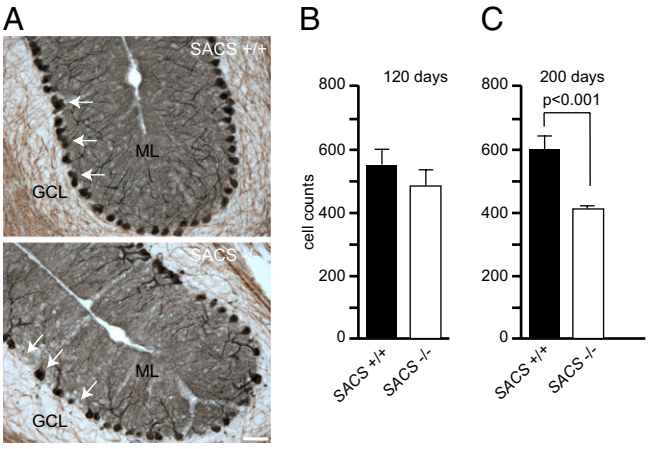

Fig. 1. Sacsin KO mice display an age-dependent loss of cerebellar Purkinje cells. (*A*) Immunohistochemical sections of cerebellum from 120-d-old sacsin KO mice (*SACS* $^{-/-}$) and wild-type littermates (*SACS*$^{+/+}$) stained with antibody against calbindin D-28K (dark-gray) to highlight Purkinje cells. Arrows point to Purkinje cell bodies or regions in which Purkinje cells are absent. GCL, granule cell layer; ML, molecular layer. (*B* and *C*) Cell counts revealing the number of Purkinje cells in cerebellum from 120-d-old (*B*) or 200-d-old (*C*) sacsin KO mice (*SACS*$^{-/-}$) and wild-type (*SACS*$^{+/+}$) littermates. Data for *B* and *C* are means ± SEM. Significance was assessed by using an unpaired Student's *t* test ($P < 0.001$). (Scale bar: 50 μm.)

Sacsin Is Localized to Mitochondria and Regulates Connectivity of Mitochondrial Networks. To explore the underlying pathophysiological defects leading to neuronal cell loss, we examined the properties of sacsin. Sacsin was demonstrated to be cytoplasmic with a mitochondrial component in SH-SY5Y cells (11). By optimizing immunostaining with antigen retrieval, we could readily detect colocalization of sacsin with mitochondrial markers in the soma, dendrites, and axons of cultured hippocampal neurons at 21 d in vitro (DIV) (Fig. 2*A*) and in COS-7 and HeLa cells (Fig. S2*A*), indicating that mitochondrial localization is a common feature in multiple cell types.

To elucidate the function of sacsin, levels of the protein were reduced by using previously validated siRNAs (11). Sacsin knockdown (KD) leads to a more interconnected mitochondrial network visualized with Mito-DsRed (Fig. S2*B*) and quantified with 3D confocal reconstructions (Fig. S2 *B* and *C*). To confirm enhanced interconnectivity, we used fluorescence recovery after photobleaching (FRAP). In the time scale used, 0–20 s, FRAP is established to provide a measure of mitochondrial continuity as opposed to mitochondrial dynamics (29–31). Mitochondria labeled with Mito-GFP demonstrate enhanced FRAP after sacsin KD (Fig. 2*B*). We next sought to determine whether similar alterations in the fusion status of mitochondria could be detected in patient samples. Two samples of fibroblasts from patients homozygous for the 8844delT mutation, the major founder mutation in Québec (1, 7), were immortalized as described (32). Western blot revealed no detectable sacsin in these cells (Fig. 2*C*), consistent with the notion that the nonsense 8844delT mutation leads to decay of the message. However, because the antibody is raised against a fragment C-terminal to the introduced stop codon, it is not possible to rule out that the lack of signal is due to a C-terminal truncation. Interestingly, both sacsin patient lines display a hyperfused mitochondrial phenotype indicated by the presence of balloon-like or bulbed mitochondria (Fig. 2 *D* and *E*). These structures are characteristic of mitochondrial hyperfusion resulting from KD of Drp1 (33, 34) or overexpression of mutant forms of Drp1 (20, 21, 35). Together, these data indicate that loss of sacsin function leads to enhanced fusion or decreased fission of mitochondria.

Further support for a potential role of sacsin in mitochondrial fission comes with the identification through coimmunoprecipitation of an interaction between the N-terminal 1,368 amino acids of sacsin and Drp1 (Fig. 2*F*). Similar to sacsin KD, Drp1 disruption leads to excessively interconnected mitochondria (20, 21, 33–35). Drp1 is found in the cytosol but also accumulates adjacent to and at the tips of mitochondria and in foci that represent potential future sites of fission (20, 21, 33). Drp1 foci are found in proximity to sacsin with partial overlap (Fig. S2*D*). Together, these data demonstrate that disruption of sacsin function leads to a more interconnected mitochondrial network, possibly through disruption of fission.

Sacsin Regulates Mitochondrial Function. Fragmentation of mitochondria isolates damaged segments for degradation and is thus an important aspect of mitochondrial quality control (31). Because sacsin KD leads to a more interconnected mitochondrial network, it is possible that the mitochondria will be functionally impaired. Tetramethylrhodamine methyl ester (TMRM) is a potential-sensitive dye used to measure mitochondrial membrane potential ($\Delta\psi_m$), which is generated by oxidative phosphorylation and is thus an indicator of mitochondrial function (34, 35). At the steady state, sacsin KD cells have a moderate but significant decrease in TMRM fluorescence compared with control (Fig. 3*A*), suggesting impaired mitochondrial function.

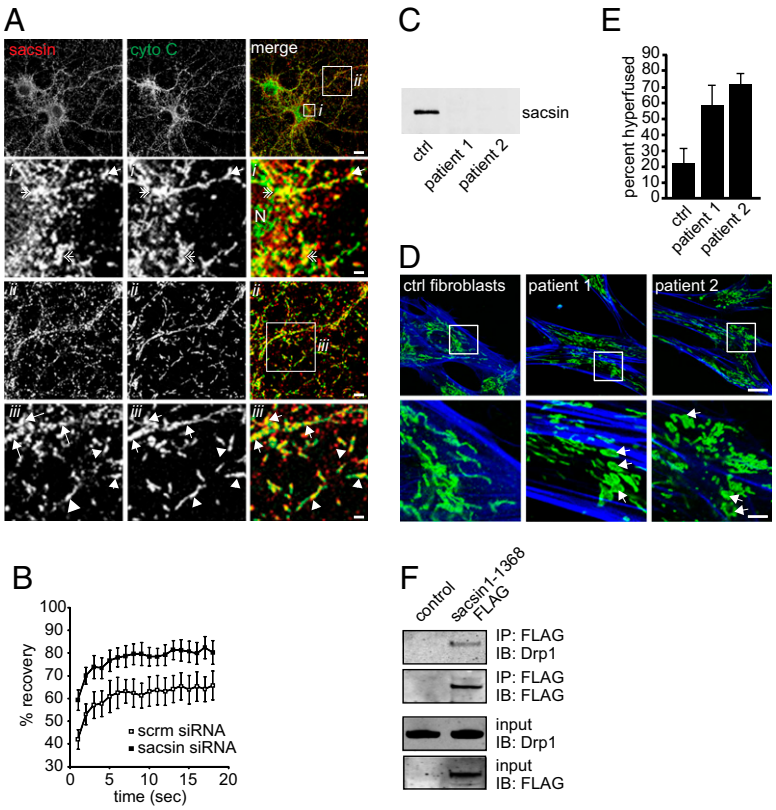

Fig. 2. Sacsin localizes to mitochondria and regulates mitochondrial morphology. (*A*) Immunofluorescence panels of cultured hippocampal neurons double-labeled with rabbit antibody against sacsin and mouse monoclonal antibody against cytochrome C (cyto C). Images in *Ai* and *Aii* are 10× and 4× zooms, respectively, of the indicated areas from *A Top*. Images in *Aiii* are 2.5× zooms from the indicated area in *Aii*. N, nucleus. Double arrows, arrows, and arrowheads indicate sacsin/cytochrome C colocalization in the soma, dendrites, and axons, respectively. (*B*) SH-SY5Y cells treated with scrambled (scrm) siRNA or siRNA targeting sacsin were transfected with pAcGFP1-Mito, and defined 2×2 μm regions of interest were bleached by using a 488-nm laser line. Recovery was monitored over 20 cycles of imaging with a 1-s interval. Points represent mean ± SEM of FRAP measurements from 32 cells from two independent experiments. (*C* and *D*) Control fibroblasts and fibroblasts from two ARSACS patients were prepared for Western blot with polyclonal antibody against sacsin (*C*), or cells were fixed and processed for immunofluorescence with antibody against TOM20 (green) to reveal mitochondria and with phalloidin Alex647 to stain actin filaments, outlining the cells (*D*). The areas boxed in *D Upper* are indicated in a 4× magnification in *D Lower*. Arrows indicated balloon-like or bulbed mitochondria, characteristic of a hyperfused phenotype (30, 31, 33). (*E*) Minimally 15 fields (containing from one to five cells) from each cell type from each of three independent experiments were coded and counted blind and independently by two individuals. The combined assessment of the hyperfused status is presented as a mean and SD. (*F*) SH-SY5Y cells were mock-transfected (control) or were transfected with a FLAG-tagged construct encoding residues 1–1368 of sacsin. Immunoprecipitation (IP) was performed with anti-FLAG antibody, and immunoblots (IB) were performed with anti-FLAG or anti-Drp1 antibodies as indicated. The inputs of FLAG-sacsin and endogenous Drp1 are indicated. (Scale bars: *A Top,* 20 μm; *Ai*, 2 μm; *Aii,* 5 μm; *Aiii,* 2 μm; *D Upper,* 10 μm; *D Lower,* 2.5 μm.)

To test further, we labeled cells with $\Delta\psi_m$-sensitive MitoTracker and then treated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which disrupts oxidative metabolism and $\Delta\psi_m$, leading to a complete loss of MitoTracker fluorescence (36, 37). With CCCP washout, MitoTracker fluorescence recovery is significantly slower in sacsin KD cells (Fig. 3*B*), suggesting reduced proton pumping by the respiratory chain or increased proton leak. We also examined whether mitochondrial dysfunction occurs in Purkinje neurons from sacsin KO mice. Organotypic cerebellar slice cultures from control and KO animals were labeled with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a membrane permeable dye that emits in the green spectra in mitochondria with a low $\Delta\psi_m$ but fluoresces red in the presence of a large $\Delta\psi_m$. Cultures from control animals have a higher red: green ratio than KO littermates (Fig. 3*C* and Fig. S3). This result is best observed in the merged and isosurface rendered panels in Fig. S3 and is highly significant (Fig. 3*C*). Taken together, our data indicate that sacsin is required for normal mitochondrial morphology and function.

Sacsin Regulates Mitochondrial Localization in Neurons. Defects in both fusion and fission are known to alter mitochondrial transport. For example, disrupting fission through expression of dominant-negative Drp1 in cultured hippocampal neurons or through KO in mice leads to the depletion of mitochondria from dendrites (38, 39). We thus hypothesized that loss of sacsin would also disrupt mitochondrial localization to dendrites. Small inhibitory RNA sequences specific for rat sacsin were prepared in a miRNA backbone (shRNAmiR) and packaged in lentivirus. This system has been used successfully in neurons and nonneuronal cells (40, 41). Several sequences lead to effective KD in hippocampal cultures, and two were selected (6034 and 14181) that cause near complete KD as determined by Western blot (Fig. 4*A*). The sequences also decrease sacsin immunofluorescence (Fig. S4 *A* and *B*), confirming the specificity of the antibody. In control neurons, mitochondria are observed in cell bodies and are distributed along the length of the dendrites (Fig. 4*B*). In sacsin KD neurons, mitochondria are clustered and accumulate in the soma and proximal dendrites (Fig. 4*B*). These data suggest that loss of sacsin function reduces the transport of mitochondria into dendrites, although additional studies will be required to examine mitochondrial transport directly.

Sacsin Is Required for Normal Dendritic Morphology. Mitochondria are essential for localized production of ATP, and they function

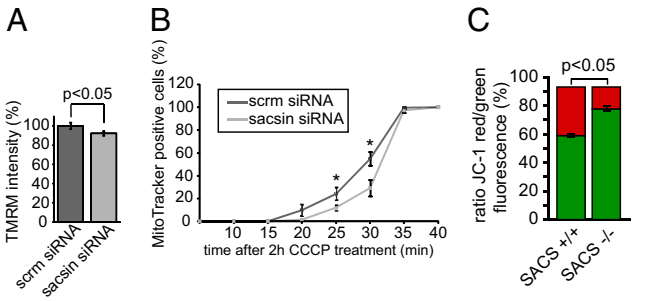

Fig. 3. Sacsin KO impairs mitochondrial function. (*A*) SH-SY5Y cells were treated with control, scrambled (scrm) siRNA, or siRNA targeting sacsin and were then incubated with TMRM. Maximum intensity projections were generated from Z-stacks, and TMRM fluorescent intensities in individual cells were quantified. Bars represent mean ± SEM. Fluorescence was quantified in 45 cells from three independent experiments. Statistical significance was assessed by using a two-tailed independent *t* test. $*P < 0.05$. (*B*) SH-SY5Y cells treated as in *A* were incubated with 20 μM CCCP for 2 h before washout. $\Delta\psi_m$-sensitive MitoTracker-Red was included in the media at 250 μM. Cells were fixed at 5-min intervals after CCCP washout and were imaged. Points represent the mean number of MitoTracker-positive transfected cells ± SEM. Cells were counted blind to experimental status in five randomly selected fields (each containing ≈30 transfected cells) from each of three independent experiments. Statistical significance was assessed by using a two-tailed independent Student's *t* test, $*P < 0.05$. (*C*) In mature cerebellar slice cultures (DIV14+), KO animals (SACS $^{-/-}$) show a significant decrease in mitochondrial membrane potential compared with wild-type littermates (SACS$^{+/+}$) as indicated by a decrease in the red/green fluorescence ratio of JC-1, a cationic dye that exhibits green emission (peak of 525 nm) in weakly polarized mitochondria that shifts to 590 nm (red) in more strongly depolarized mitochondria.

in calcium buffering (16, 42). As a result, alterations in mitochondrial transport and distribution in neurons can lead to defects in neuronal development (43). For example, neurons from mice or flies with disrupted Drp1 function accumulate mitochondria in cell bodies, have reduced numbers of neurites per neuron, and altered synaptic function (39, 44). To determine

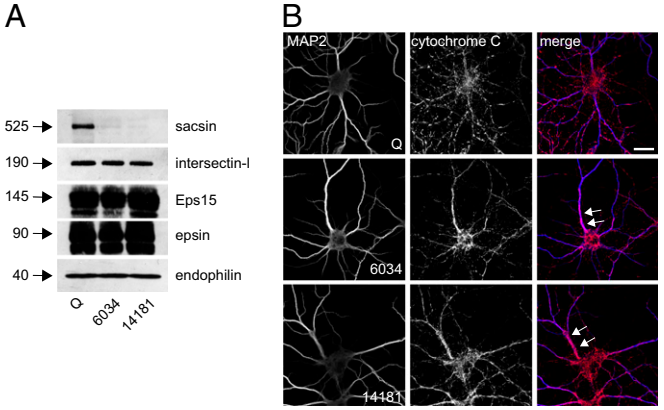

Fig. 4. Sacsin loss of function alters the distribution of mitochondria in neurons. (*A*) Western blots (using antibodies against the indicated proteins) of lysates from cultured hippocampal neurons transduced with lentivirus encoding a control shRNAmiR (Q) or one of two different shRNAmiRs targeting sacsin (6034, 14181). The molecular mass of the proteins is indicated on the left. (*B*) Immunofluorescence panels of cultured hippocampal neurons (DIV14) double-labeled with chicken polyclonal antibody against MAP2 (blue) and mouse monoclonal antibody against cytochrome C (red). At DIV4, the neurons were transduced with lentivirus encoding a control shRNAmiR (Q) or one of two different shRNAmiRs targeting sacsin (6034, 14181). Arrows indicate mitochondria accumulating in proximal dendrites. (Scale bar: 40 μm.)

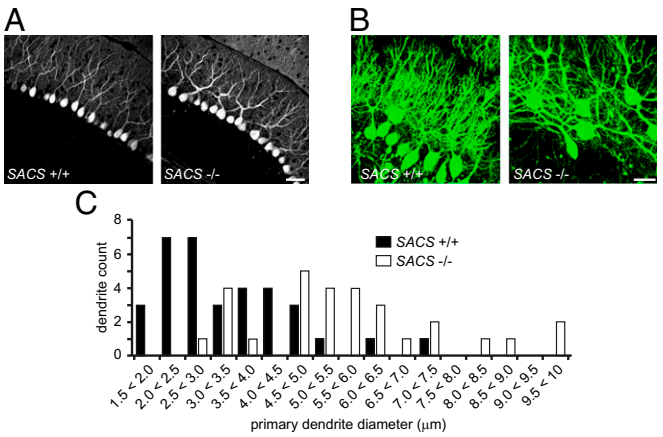

Fig. 5. Sacsin KO alters dendritic morphology. (*A*) Immunofluorescence panels of sections from cerebellum of 120-d-old sacsin KO mice (*SACS* $^{-/-}$) and control littermates (*SACS*$^{+/+}$) stained with antibody against calbindin to reveal Purkinje cells. (*B*) Immunofluorescence panels of cerebellar slice cultures from sacsin KO mice (*SACS*$^{-/-}$) and control littermates (*SACS*$^{+/+}$) stained with antibody against calbindin to reveal Purkinje cells. (*C*) Quantification of images as in *B* reveals that the distribution of proximal dendrite diameter is shifted to larger diameters in *SACS* $^{-/-}$ neurons. The mean proximal dendrite diameter of *SACS*$^{-/-}$ Purkinje neurons is significantly larger than those from SACS$^{+/+}$ littermates (5.58 ± 1.86 μm vs. 3.25 ± 1.06 μm; $P < 0{,}001$; two-tailed independent Student's *t* test). (Scale bars: 50 μm.)

whether similar processes occur after loss of sacsin function, we examined dendritic morphology in cultured hippocampal neurons. Sacsin KD neurons displayed fewer dendrites per neuron compared with control (Fig. S5 *A* and *B*). The remaining dendrites in sacsin KD neurons are significantly thicker (Fig. S5*C*), yet the overall length of any given dendrite is unaltered (Fig. S5*D*). Importantly, Purkinje cells in 120-d-old sacsin KO mice, thus before significant neuronal loss, also display disordered, tortuous dendritic fields compared with control littermates (Fig. 5*A*). All areas of the cerebellum of 120-d-old mice show the same general modification in dendritic morphology. Similar results are seen in organotypic cerebellar slice cultures from KO mice, in which the dendritic fields of the Purkinje cells appear highly disordered (Fig. 5*B*). The dendrites also appear to be thicker (Fig. 5*B*), and quantification of these cultures revealed significant thickening of primary dendrites (Fig. 5*C*). Thus, sacsin loss of function disrupts dendritic morphology, perhaps due to alterations in mitochondrial delivery to dendrites.

Discussion

ARSACS was first identified as a regional disease in Québec, and the majority of Québec patients are homozygous for an 8844delT nonsense founder mutation (1, 7). However, there are now >100 different nonsense and missense mutations that have been identify in the *SACS* gene worldwide, most of them being in the large exon 10 (5, 8–10). The clinical spectrum of ARSACS has also broadened in recent years because some patients outside of Québec present mild nonspastic ataxia to severe spastic ataxia with mental retardation (10). Moreover, sacsin has been linked to other neurological disorders. It is a node in an ataxia interactome, sharing interacting partners with other ataxia proteins including ataxin-1, ataxin-3, and frataxin (45). Interestingly, sacsin is a major target for the ubiquitin ligase Ube3a that is nonfunctional in Angelman's syndrome, a severe neurological disorder with myriad symptoms including intellectual disability and ataxic movements, and it is hypothesized that changes in sacsin function account for the motor symptoms of this disease (14, 46). However, despite the seeming importance of the sacsin

protein, there has been little progress regarding the cell biological role of sacsin and the underlying pathology in ARSACS.

Here, we demonstrate that sacsin localizes to mitochondria and is required for normal mitochondrial function, morphology, and localization in neurons. Moreover, the *SACS* KO mouse model reveals age-dependent loss of Purkinje cells, consistent with the progressive ataxia seen in patients. It is likely however that cerebellar dysfunction precedes loss of Purkinje cells. Purkinje cells transform excitatory afferent signals to inhibitory efferent signals that target the neurons of the deep cerebellar nuclei and vestibular nuclei. These pathways are a prerequisite for normal motor function, and even minor disturbances cause uncoordinated movements and ataxia (47). The *SACS* KO mice display striking alterations in the dendritic fields of cerebellar Purkinje cells that precede cell death. Numerous alterations in cell function including improper mitochondrial transport can lead to abnormal dendritic morphology and altered dendritic fields of Purkinje cells are often seen in mouse models of neurodegenerative diseases featuring ataxia (43, 48). In fact, neuronal and synaptic dysfunctions are observed before cell death in both patients and animal models of Huntington (49), Alzheimer (50), and Parkinson (51). It will be important to determine whether there are changes in synaptic connectivity and function in the *SACS* KO mice.

Another commonality between these major neurodegenerative diseases and ARSACS is mitochondrial involvement. More specifically disruptions in mitochondrial dynamics and transport contribute to the disease process (24, 27, 52). Here, we demonstrate that sacsin localizes to mitochondria and that loss of sacsin function alters the balance between fusion and fission, possibly through disruption of Drp1 function. However, one must interpret this possibility with caution given that alterations in mitochondria morphology are relatively common in response to disruption of mitochondrial proteins and other cellular stressors including oxidative stress (53). Moreover, sacsin has multiple domains related to protein quality control (11–15). Thus, after the loss of sacsin function, alterations in mitochondrial proteostasis could account directly for the observed defects in mitochondrial function, with morphological changes as a consequence. Regardless, these changes lead to altered distribution of mitochondria in cultured neurons and Purkinje cells of KO mice. We propose that altered mitochondrila localization contributes to the observed alterations in the morphology and function of dendrites, ultimately causing cell death. Our data thus allow us to target mitochondrial repair in disease therapy.

Materials and Methods

KO mice were generated by replacing most of exon 10 of *SACS* with an IRES-βGal cassette. Production and use of lentivirus to drive shRNAmiRs for KD in primary hippocampal cultures were described (40, 41). Culture and analysis of SH-SY5Y cells after sacsin KD with siRNA were described (11). Detailed information regarding these procedures and reagents and protocols for immunofluorescence, immunocytochemistry, live cell studies, generation of ARSACS patient cell lines, generation and analysis of organotypic slice cultures, Western blotting, and immunoprecipitation are described in *SI Materials and Methods*.

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1. Bouchard JP, Barbeau A, Bouchard R, Bouchard RW (1978) Autosomal recessive spastic ataxia of Charlevoix-Saguenay. *Can J Neurol Sci* 5:61–69.
2. Bouchard JP (1991) Recessive spastic ataxia of Charlevoix-Saguenay. *Hereditary Neuropathies and Spinocerebellar Atrophies Handbook of Clinical Neurology*, ed de Jong JMBV (Elsevier, Amsterdam), pp 451–459.
3. Dupré N, Bouchard JP, Brais B, Rouleau GA (2006) Hereditary ataxia, spastic paraparesis and neuropathy in the French-Canadian population. *Can J Neurol Sci* 33: 149–157.
4. Martin MH, Bouchard JP, Sylvain M, St-Onge O, Truchon S (2007) Autosomal recessive spastic ataxia of Charlevoix-Saguenay: A report of MR imaging in 5 patients. *AJNR Am J Neuroradiol* 28:1606–1608.
5. Bouhlal Y, Amouri R, El Euch-Fayeche G, Hentati F (2011) Autosomal recessive spastic ataxia of Charlevoix-Saguenay: An overview. *Parkinsonism Relat Disord* 17:418–422.
6. Bouchard JP, Richter A, Melancon SB, Mathieu J, Michaud J (2000) Autosomal recessive spastic ataxia (Charlevoix-Saguenay). *Handbook of Ataxia Disorders*, ed Klockether T (Marcel Dekker, New York), pp 312–324.
7. Engert JC, et al. (2000) ARSACS, a spastic ataxia common in northeastern Québec, is caused by mutations in a new gene encoding an 11.5-kb ORF. *Nat Genet* 24:120–125.
8. Gomez CM (2004) ARSACS goes global. *Neurology* 62:10–11.
9. Vermeer S, et al. (2008) ARSACS in the Dutch population: A frequent cause of early-onset cerebellar ataxia. *Neurogenetics* 9:207–214.
10. Baets J, et al. (2010) Mutations in SACS cause atypical and late-onset forms of ARSACS. *Neurology* 75:1181–1188.
11. Parfitt DA, et al. (2009) The ataxia protein sacsin is a functional co-chaperone that protects against polyglutamine-expanded ataxin-1. *Hum Mol Genet* 18:1556–1565.
12. Anderson JF, Siller E, Barral JM (2010) The sacsin repeating region (SRR): A novel Hsp90-related supra-domain associated with neurodegeneration. *J Mol Biol* 400: 665–674.
13. Anderson JF, Siller E, Barral JM (2011) The neurodegenerative-disease-related protein sacsin is a molecular chaperone. *J Mol Biol* 411:870–880.
14. Greer PL, et al. (2010) The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140:704–716.
15. Kozlov G, et al. (2011) Structural basis of defects in the sacsin HEPN domain responsible for autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS). *J Biol Chem* 286:20407–20412.
16. McBride HM, Neuspiel M, Wasiak S (2006) Mitochondria: More than just a powerhouse. *Curr Biol* 16:R551–R560.
17. Chen H, Chan DC (2010) Physiological functions of mitochondrial fusion. *Ann N Y Acad Sci* 1201:21–25.
18. Reddy PH, et al. (2011) Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. *Brain Res Brain Res Rev* 67:103–118.
19. Otsuga D, et al. (1998) The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J Cell Biol* 143:333–349.
20. Smirnova E, Shurland DL, Ryazantsev SN, van der Bliek AM (1998) A human dynamin-related protein controls the distribution of mitochondria. *J Cell Biol* 143:351–358.
21. Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* 12:2245–2256.
22. Westermann B (2010) Mitochondrial fusion and fission in cell life and death. *Nat Rev Mol Cell Biol* 11:872–884.
23. Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 443:787–795.
24. Chen H, Chan DC (2009) Mitochondrial dynamics—fusion, fission, movement, and mitophagy—in neurodegenerative diseases. *Hum Mol Genet* 18(R2):R169–R176.
25. Cho DH, Nakamura T, Lipton SA (2010) Mitochondrial dynamics in cell death and neurodegeneration. *Cell Mol Life Sci* 67:3435–3447.
26. Su B, et al. (2010) Abnormal mitochondrial dynamics and neurodegenerative diseases. *Biochim Biophys Acta* 1802:135–142.
27. Schon EA, Przedborski S (2011) Mitochondria: The next (neurode)generation. *Neuron* 70:1033–1053.
28. Gazulla J, et al. (2011) Is the ataxia of Charlevoix-Saguenay a developmental disease? *Med Hypotheses* 77:347–352.
29. Mitra K, Wunder C, Roysam B, Lin G, Lippincott-Schwartz J (2009) A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc Natl Acad Sci USA* 106:11960–11965.
30. Mitra K, Lippincott-Schwartz J (2010) Analysis of mitochondrial dynamics and functions using imaging approaches. *Curr Protoc Cell Biol* 4:4.25.1–21.
31. Tanaka A, et al. (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J Cell Biol* 191:1367–1380.
32. Lochmüller H, Johns T, Shoubridge EA (1999) Expression of the E6 and E7 genes of human papillomavirus (HPV16) extends the life span of human myoblasts. *Exp Cell Res* 248:186–193.
33. Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell* 15:5001–5011.
34. Estaquier J, Arnoult D (2007) Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. *Cell Death Differ* 14: 1086–1094.
35. Frank S, et al. (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 1:515–525.
36. Ishihara N, Jofuku A, Eura Y, Mihara K (2003) Regulation of mitochondrial morphology by membrane potential, and DRP1-dependent division and FZO1-dependent fusion reaction in mammalian cells. *Biochem Biophys Res Commun* 301:891–898.

37. Geisler S, et al. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 12:119–131.
38. Li Z, Okamoto K, Hayashi Y, Sheng M (2004) The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* 119:873–887.
39. Ishihara N, et al. (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nat Cell Biol* 11:958–966.
40. Katoh Y, et al. (2009) The clavesin family, neuron-specific lipid- and clathrin-binding Sec14 proteins regulating lysosomal morphology. *J Biol Chem* 284:27646–27654.
41. Allaire PD, et al. (2010) The Connecdenn DENN domain: A GEF for Rab35 mediating cargo-specific exit from early endosomes. *Mol Cell* 37:370–382.
42. MacAskill AF, Kittler JT (2010) Control of mitochondrial transport and localization in neurons. *Trends Cell Biol* 20:102–112.
43. MacAskill AF, Atkin TA, Kittler JT (2010) Mitochondrial trafficking and the provision of energy and calcium buffering at excitatory synapses. *Eur J Neurosci* 32:231–240.
44. Verstreken P, et al. (2005) Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. *Neuron* 47:365–378.
45. Lim J, et al. (2006) A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. *Cell* 125:801–814.
46. Mabb AM, Judson MC, Zylka MJ, Philpot BD (2011) Angelman syndrome: Insights into genomic imprinting and neurodevelopmental phenotypes. *Trends Neurosci* 34:293–303.
47. Sotelo C, Alvarado-Mallart RM (1991) The reconstruction of cerebellar circuits. *Trends Neurosci* 14:350–355.
48. Dusart I, Guenet JL, Sotelo C (2006) Purkinje cell death: Differences between developmental cell death and neurodegenerative death in mutant mice. *Cerebellum* 5:163–173.
49. Milnerwood AJ, Raymond LA (2010) Early synaptic pathophysiology in neurodegeneration: Insights from Huntington's disease. *Trends Neurosci* 33:513–523.
50. Palop JJ, Mucke L (2010) Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: From synapses toward neural networks. *Nat Neurosci* 13:812–818.
51. Nakamura K, Edwards RH (2007) Physiology versus pathology in Parkinson's disease. *Proc Natl Acad Sci USA* 104:11867–11868.
52. Wang X, et al. (2009) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. *J Neurosci* 29:9090–9103.
53. Ichishita R, et al. (2008) An RNAi screen for mitochondrial proteins required to maintain the morphology of the organelle in Caenorhabditis elegans. *J Biochem* 143: 449–454.